Appendix C

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant	:	Remacle et al.
Appl. No.	:	10/606,162
Filed	:	June 25, 2003
For	:	METHOD FOR THE DETERMINATION OF CELL ACTIVATION
Examiner	:	Petersen, Clark D.
Group Art Unit	:	1657

DECLARATION UNDER 37 C.F.R §1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

1. This Declaration is being submitted to demonstrate the advantages resulting from evaluating an activation state of cell by quantifying the level of phosphorylation of at least three cellular proteins of the same or different pathways present in the same cell extract and the lack of these advantages in the methods described in the cited Schooler reference.

2. I am an inventor on the above-identified patent application and am familiar with the specification and prosecution history.

3. I have extensive experience in the field of the claimed invention as indicated in the attached Curriculum Vitae provided herewith as Exhibit A.

4. In the claimed methods, an activation state of cells in the same cell extract is generated from signals obtained on two distinct arrays, the first array being dedicated to the detection of at least three phosphorylated cellular proteins and the second array being dedicated to the detection of at least three phosphorylated and non-phosphorylated cellular proteins. We conducted experiments to assess the dependence of obtaining an activation state of a biological sample by

quantifying the phosphorylation level of at least three cellular proteins belonging to two different pathways.

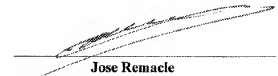
Exhibit 1 represents the four regulation pathways of the Mitogen activated protein kinases (MAPK). The four cascades are activated by multiple agents. If one looks at only one activated TF, it is impossible to predict which activation profile is obtained in given stimulation condition because (i) each TF can be activated by multiple MAPK; (ii) each MAPK can activate multiple TFs and (iii) the four pathways can produce additive or antagonist effects. Therefore, if a TF belonging to an activation cascade is not activated, it does not mean that a cascade is not activated.

Exhibit 2 attached hereto shows signals of three phosphorylated versus phosphorylated and non phosphorylated transcription factors (TFs). Exhibit 3 (attached hereto) shows a quantification of the results shown in Exhibit 2 and the ratio between the phosphorylated versus phosphorylated and non phosphorylated TFs present in the cell extract. Extracts were obtained from two test conditions, i.e. Hela cells stimulated with PMA for either 10 min or 1 hour.

As illustrated in the accompanying Exhibits, one cannot predict an activation state of cells if the phosphorylation level of only one TF is analyzed. As shown in Exhibit 3, analyzing the phosphorylation of c-Jun only would suggest no difference between the two stimulation times. Simultaneously analyzing the phosphorylation of c-Jun, ATF-2 and Elk-1 however shows a strong influence of the stimulation time on cellular activation. Similarly, analyzing the phosphorylation of Elk-1 only shows an increase in the TF activation after 1h PMA stimulation, compared to 10 min. Since Elk-1 can be activated by three cascades, namely the ERK1/2, JNK and p38 cascades, it is not possible, based on this sole result, to determine which cascade(s) is (are) activated. The dual analysis of Elk-1 and ATF-2 phosphorylation shows that at least the p38 and/or JNK cascades are activated, but this analysis still cannot answer the question. The triple analysis of the Elk-1, ATF-2 and c-Jun phosphorylation shows that the JNK cascade is not activated, because this is the only cascade leading to c-Jun phosphorylation, ands no c-Jun phosphorylation is observed. Therefore, the cascade leading to ATF-2 phosphorylation is, by deduction, p38, and the cascades activating Elk-1 are JNK1/2 and p38.

5. Schooler et al. describe assays for determining the activation of a unique protein (EGFR) in an ELISA assay. Microtiter dishes were coated with anti-EGFR monoclonal antibodies to capture the receptor followed by parallel detection of receptor and phosphotyrosine content with secondary antibodies. The ratio of these two parameters reflects the protein activation. It is the activity of a single protein which is determined and not the activation state of cells as provided by the invention. The method of School et al. is not adapted to quantify the phosphorylation level of more than one protein, as one well of the microtiter plate contains only one type of capture antibody. The phosphorylated EGFR and EGFR phosphorylated and non phosphorylated forms are recognized in separated wells of the microtiter plate. Performing assays on the evaluation of the activity of several proteins in independent wells would introduce some variability in the quantification of the phosphorylation level of different proteins. Schooler et al. do not mention nor suggest that the method would be useful for the evaluation of the phosphorylation level of several proteins simultaneously in the same cell extract, nor that arrays of capture antibodies could be used for the evaluation of such phosphorylation level. In contrast to the teaching of Schooler et al. the present invention enables to assay different proteins in the same cell extract on the same surface as provided by the array of capture molecules.

7. I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful, false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or patent issuing therefrom.

Dated: 15 May 2007 By: 
Jose Remacle

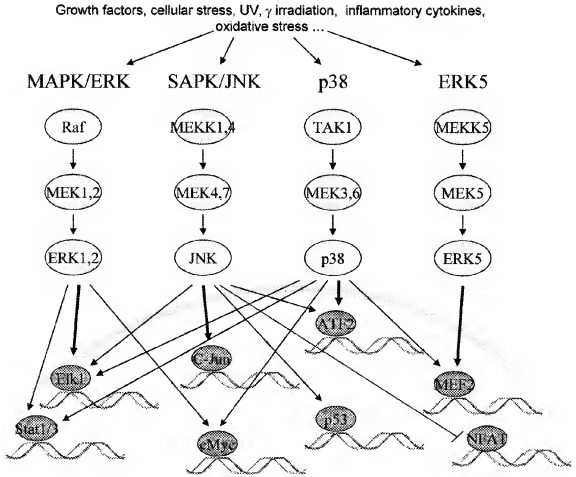


Exhibit 1: The four regulatory circuits of the MAPK: ERK1/2, JNK, p38 and ERK5. The four cascades are activated by multiple agents like growth factors, cellular stress, UV, gamma irradiation, inflammatory cytokines, oxidative stress, mitotic agents (like PMA), etc.

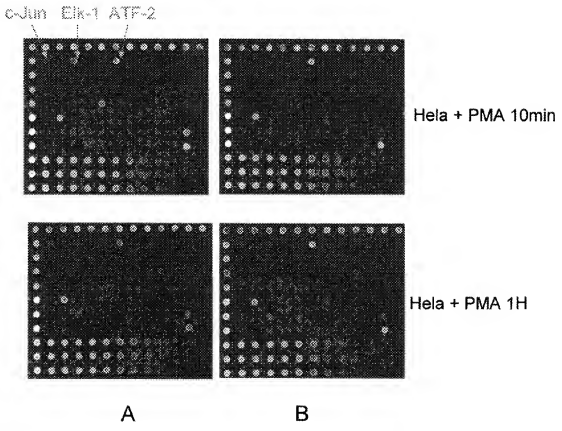
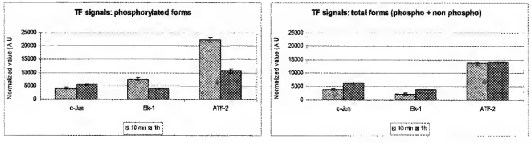


Exhibit 2: Signals of 3 phosphorylated versus phosphorylated and non phosphorylated transcription factors (TFs) in nuclear extracts of Hela cells after PMA stimulation for 10 min or 1 hour using the TF Chip MAPK kit (Eppendorf; Germany). Spots of the array are present in triplicate; one spot corresponding to c-Jun, Elk-1 and ATF-2 are shown. Top and left spots are positive controls; Spots corresponding to each TF contain double-stranded DNA comprising a specific binding site for the TF and a common spacer.
Two slides (two arrays each) were processed in parallel: one slide was contacted with 30 µg nuclear extract of Hela cells + PMA (10 min stimulation) and the other slide with 30 µg nuclear extract of Hela cells + PMA (1 hour stimulation). The phosphorylation of 3 TFs (c-Jun, Elk-1 and ATF-2) was measured on the left arrays (A) using a mix of 3 antibodies respectively directed to phospho-c-Jun, phospho-Elk-1 and phospho-ATF-2, while the total TFs (phosphorylated and non phosphorylated forms) were detected on the right arrays (B) using a mix of antibodies against c-Jun, Elk-1 and ATF-2. Signals were obtained with Cy3-labeled secondary antibodies and fluorescence scanning using a ScanArray Express microarray scanner from Packart BioScience and a laser power of 100. Scans were performed with a gain = 80.

A



B

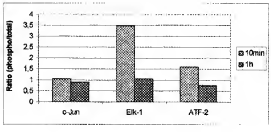


Exhibit 3: A. Quantification of signals from Exhibit 2. Y axis represents the TF signals as obtained from the data analysis software for TF Chip microarrays (TF Chip MAPK kit, Eppendorf, Germany).
B; Ratio between the phosphorylated versus phosphorylated + non phosphorylated TFs.